



# Inhibition of Axl improves the targeted therapy against ALK-mutated neuroblastoma



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## ABSTRACT

Neuroblastoma (NB) patients harboring mutated ALK can be expected to potentially benefit from targeted therapy based on ALK tyrosine kinase inhibitor (TKI), such as crizotinib and ceritinib. However, the effect of the treatment varies with different individuals, although with the same genic changes. Axl receptor tyrosine kinase is expressed in a variety of human cancers, but little data are reported in NB, particularly in which carrying mutated ALK. In this study, we focus on the roles of Axl in ALK-mutated NB for investigating rational therapeutic strategy. We found that Axl is expressed in ALK-positive NB tissues and cell lines, and could be effectively activated by its ligand GAS6. Ligand-dependent Axl activation obviously rescued crizotinib-mediated suppression of cell proliferation in ALK-mutated NB cells. Genetic inhibition of Axl with specific small interfering RNA markedly increased the sensitivity of cells to ALK-TKIs. Furthermore, a small-molecule inhibitor of Axl significantly enhanced ALK-targeted therapy, as an increased frequency of apoptosis was observed in NB cells co-expressing ALK and Axl. Taken together, our results demonstrated that activation of Axl could lead to insensitivity to ALK inhibitors, and dual inhibition of ALK and Axl might be a potential therapeutic strategy against ALK-mutated NB.

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## 1. Introduction

As the most common extracranial solid tumor, neuroblastoma (NB) is most commonly diagnosed in early childhood, accounting for 10% of all pediatric oncology deaths [1]. Increasing evidences have showed that activating mutations in the anaplastic lymphoma kinase (ALK) gene are considered to be the leading cause for most cases of hereditary neuroblastoma [2]. Activated ALK caused by ALK rearrangements, such as NPM-ALK, has been shown to influence several main signaling pathways, thus promoting cell growth and survival [3]. However, the situation appears to be more complex in neuroblastoma as mutations and amplification in ALK gene both result in constitutive phosphorylation, leading to oncogenic effects [4].

NB with ALK aberration (mutation or amplification) have been implied significantly more sensitive to ALK inhibitors than that with wild-type ALK. ALK is mutated in 8% of diagnostic tumor

samples [5], and amplification of the ALK gene has only been described in 1.2–4.4% of NB patients [2,6]. In this study, we focused on the therapeutic effect of ALK-targeted therapy in ALK-mutated neuroblastoma. Growing evidences suggest that patients with ALK-mutated neuroblastoma might benefit from treatment with ALK tyrosine kinase inhibitors (TKIs), such as crizotinib and ceritinib, which have been approved by the US Food and Drug Administration (FDA) in 2011 and April this year [7,8].

Receptor tyrosine kinase Axl is a member of the TAM (Tyro3/Axl/Mer) family, and has reported been associated with a spectrum of human cancers [8]. Numerous studies have revealed that the oncogenic potential of Axl is attributed to the anti-apoptotic and proliferative signaling pathways triggered by activation of the tyrosine kinase domain [9,10]. Ligands that can induce Axl autophosphorylation have been described, such as GAS6 and protein S, potentially resulting in the activation of several canonical oncogenic signaling pathways [11]. Recent studies have shown that activation of Axl mediates resistance to chemotherapeutic drugs [12], as well as targeted therapy based on small-molecule inhibitor, such as erlotinib and lapatinib [13,14]. However, the expression and functional consequences of Axl expression in neuroblastoma and the role in targeted therapy against neuroblastoma by ALK-TKIs are poorly understood.

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Here, we focus on the role of Axl in ALK-mutated neuroblastoma, and provide support for this protein as attractive novel therapeutic target to design relevant therapeutic strategies for targeted therapy against ALK-mutated neuroblastoma patients.

## 2. Materials and methods

### 2.1. Tissue samples and immunohistochemistry (IHC)

The collection of tumor tissues from NB patients was approved by our Institutional Review Board (IRB). For IHC, the tissue samples from consenting patients were cut in 5- $\mu$ m sections. After antigen retrieval, the samples were incubated overnight using a primary antibody, anti-ALK (Cell Signaling Technology; Denver, MA, USA) and anti-Axl (Cell Signaling Technology). Then, signal was detected using a suitable HRP-labeled second antibody with DAB as the chromagen (Dako, Carpinteria, CA). The staining was photographed under an inverted light microscope (Olympus, Tokyo, Japan).

### 2.2. Cell culture and reagents

A panel of ALK-positive NB cell lines (NB1643, Kelly, SHSY5Y and NBSD) were maintained in DMEM (Invitrogen, Breda, The Netherlands) containing 10% heat inactivated fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) and 100 Units/ml penicillin, 100 mg/ml streptomycin (Hyclone Laboratories, Logan, UT, USA). NSCLC cell line H2228, which harbors the fusion protein EML4-ALK, was maintained in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) supplemented with 10% FBS. ALK inhibitors crizotinib (PF-02341066) and ceritinib (LDK378) as well as Axl inhibitor BGB324 were obtained from Selleck Chemicals (Houston, TX, USA), and stock solutions were prepared in DMSO. Recombinant human GAS6 was purchased from R&D Systems (Minneapolis, MN, USA) and reconstituted in PBS.

### 2.3. Western blot analysis

Whole-cell lysates were prepared in RIPA buffer with protease inhibitors (Sigma, St. Louis, MI, USA). Antibodies for Western blot were: phospho-ALK (pALK, Tyr1507) from Abcam (Cambridge, MA, USA); phospho-Axl (pAxl, Tyr702), ALK, Axl and caspase3 Cell Signaling Technology (Denver, MA, USA).

### 2.4. Cell proliferation assay

Cells were seeded in starvation medium in 96-well plates in a volume of 100  $\mu$ l medium. After 12 h, cells were treated with crizotinib (100 nM) in the presence of GAS6 (200 ng/ml) for 3 days, and the cell proliferation were determined by the MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, Wisconsin) and normalized to control. For determination of IC<sub>50</sub> values, cells were transfected with control or Axl specific small interfering RNAs using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. Cells were then cultured for another 3 days in the presence of increasing concentrations of crizotinib or ceritinib. Each experiment was repeated 4–6 times. The IC<sub>50</sub> values were analyzed using Prism 5 (Version 5, GraphPad Software, Inc.).

### 2.5. Colony formation assay

NB1643 cells were plated in 6-well dishes at 200 cells per well and cultured in medium containing crizotinib (100 nM) and GAS6 (200 ng/ml) for ten days. Colonies were washed with PBS and stained with crystal violet (0.5% w/v in 25% methanol). Stained

plates were rinsed in PBS and allowed to dry at room temperature. Colony diameter was photographed under an inverted light microscope (Olympus, Tokyo, Japan).

### 2.6. Flow cytometric analysis of apoptosis

After treated with crizotinib (100 nM), ceritinib (100 nM) and BGB324 (300 nM) for 48 h, cells were resuspended and incubated with 5  $\mu$ l FITC-conjugated annexin V (Invitrogen, Carlsbad, CA, USA) for 10 min in the dark. Then, cells were centrifuged and resuspended in 290  $\mu$ l binding buffer and auditioned with 10  $\mu$ l propidium iodide (PI) (Sigma-Aldrich). The Annexin-V-FITC and PI binding were determined using a flow cytometer (Beckman Coulter).

### 2.7. Animals and subcutaneous xenograft model

$5 \times 10^6$  NB1643 cells were resuspended in 100  $\mu$ l PBS and implanted into the back of female BALB/c nude mice. For the drug treatment, mice with a size of  $\sim 100$  mm<sup>3</sup> tumors were then treated with crizotinib alone (50 mg/kg), or BGB324 alone (125 mg/kg), or their combination. Tumor growth was measured by calipers in two dimensions, and the tumor volume was calculated as length  $\times$  width<sup>2</sup>/2. Tumor weight was also measured at the end of the study.

### 2.8. Statistical analysis

Data are expressed as mean  $\pm$  s.e.m. All data are representative of three independent experiments unless otherwise noted. The significance of differences between groups was assessed by two-tailed *t*-test or one-way ANOVA. All analyses were all other data were performed using Prism 5 (Version 5, GraphPad Software, Inc.).

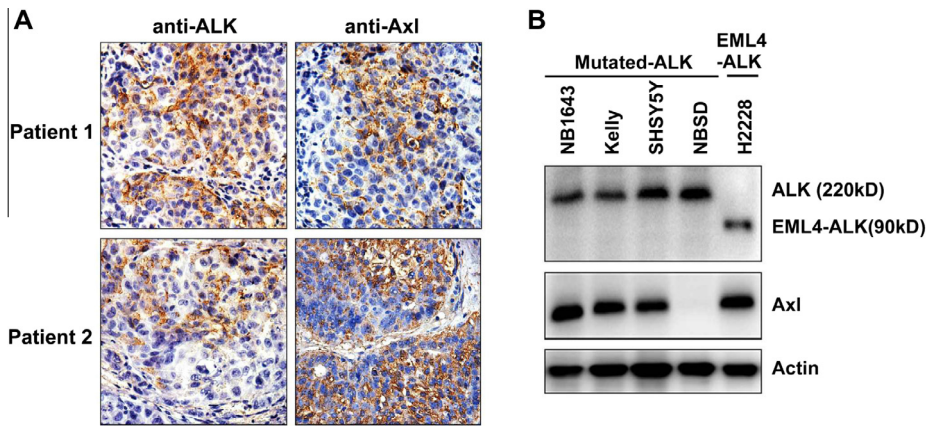
## 3. Results

### 3.1. Axl receptor tyrosine kinase is expressed in ALK-positive human neuroblastoma tissues and cell lines

Previous studies have revealed that Axl is expressed in a variety of human cancers, however, little data are reported in neuroblastoma, particularly in which expresses ALK. In this study, we focus on the roles of Axl in ALK-mutated neuroblastoma for investigating rational therapeutic strategy. We firstly examined the expression of Axl in 15 human neuroblastoma tissues by immunohistochemistry (IHC), and found that 11 tissues (73.3%) expressed Axl protein, including 2 tissues expressing ALK (Fig. 1A). We expand on that observation by analyzing Axl expression in a panel of neuroblastoma cell lines containing mutated ALK shown in Fig. 1B. Western blot analysis showed that 3 out of the 4 ALK-positive lines (75%) expressed Axl protein at varying levels (Fig. 1B), implicating the co-expression ALK and Axl in neuroblastoma. Additionally, NSCLC cell line H2228 carrying EML4-ALK fusion gene also presented a high level of Axl, indicating that this symbiotic relationship may also exist in other ALK-expressing tumors.

### 3.2. Ligand-dependent Axl activation promotes cell growth and impairs the efficiency of ALK inhibitor

Previous reports have showed that Axl can be activated by its ligand, such as GAS6, promoting multiple pro-oncogenic signaling, include pathways promoting survival, increasing migration, and inhibiting apoptosis. To investigate the role of activation of Axl by GAS6 in ALK-mutated neuroblastoma cells, the following NB cell lines were used: NB1643 (ALK mutation R1275Q), Kelly (ALK



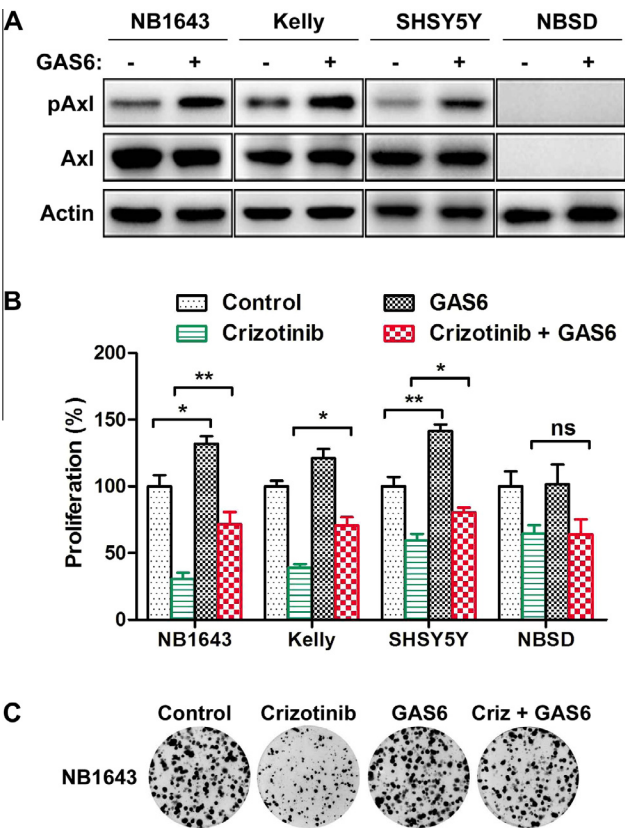
**Fig. 1.** Axl receptor tyrosine kinase is expressed in ALK-positive human neuroblastoma tissues and cell lines. (A) Immunohistochemical staining of ALK and Axl in sections of neuroblastoma. Micrographs shown represent the range of staining observed in tumor cells. (B) The expression of ALK and Axl in a panel of NB cells and NSCLC H2228 cells were examined by Western blot.

mutation F1174L), SHSY5H (ALK mutation F1174L) and NBSD (ALK mutation F1174L, but no Axl expression). In all three Axl expressing cell lines, GAS6 stimulation resulted in a greater magnitude of phosphorylated Axl (Fig. 2A), indicating the activation of Axl by GAS6 in NB cells. Since mutated ALK is a major familial neuroblastoma predisposition gene and has been considered as a potential

therapeutic target, we further explored the roles of Axl in targeted therapy with ALK inhibitors in neuroblastoma. As shown in Fig. 2B, while crizotinib (an ALK inhibitor) markedly inhibited the proliferation of all ALK-mutated cell lines, GAS6 stimulation greatly resulted in a certain degree of resistance to crizotinib by dramatically restoring the cell growth and colony-forming capacity in Axl-expressing cells (Fig. 2B and C). However, little or no such effects were observed in Axl-negative NBSD cells. Additionally, GAS6 alone also promoted cell growth in NB1643 and SHSY5Y cells. Taken together, these results suggested that ligand-activation of Axl may be involved in the insensitivity of NB to ALK-targeted therapy.

3.3. Genetic inhibition of Axl leads to increased sensitivity to ALK inhibitors

Since activation of Axl impaired the efficiency of ALK-targeted therapy, we wonder whether inhibiting Axl could further increase the sensitivity to ALK inhibitors, such as crizotinib and ceritinib, which are currently used clinically. The expression of Axl was silenced with specific small interfering RNA (si-Axl), and the



**Fig. 2.** Ligand-dependent Axl activation promotes cell growth and impairs the efficiency of ALK inhibitor. (A) Cells were treated with recombinant human GAS6 (200 ng/ml) for 6 h, and the whole-cell lysates of the indicated cell lines were subjected to Western blot analysis for the expression of total and phosphorylated Axl. (B) Cells were cultured in the absence or presence of crizotinib (100 nM) and GAS6 (200 ng/ml) for 3 days, the cell growth was examined by MTS assay. (C) NB1643 cells were seeded into 6-well dishes, and the colony-formation ability was measured in the medium containing crizotinib (100 nM) and GAS6 (200 ng/ml). \* $P < 0.05$  and \*\* $P < 0.01$  for the indicated comparisons; ns, not significant.

**Table 1**  
IC<sub>50</sub> values determined by MTS analysis.

IC <sub>50</sub> values (nM)	si-Control	si-Axl1	si-Axl2
<b>NB1643</b>			
Crizotinib	289.3	67	40.5
Ceritinib	91.8	23.6	15
<b>Kelly</b>			
Crizotinib	258.3	60.5	32.6
Ceritinib	83.4	18.4	12.2
<b>SHSY5Y</b>			
Crizotinib	538.3	72	41.9
Ceritinib	222.2	17.7	11.7
<b>NBSD</b>			
Crizotinib	484.2	435.9	464.2
Ceritinib	200.8	148.4	152.6
<b>H2228</b>			
Crizotinib	703.5	107	52.2
Ceritinib	155.2	33.7	17.8

*Note:* After transfection with control (si-Control) or Axl-specific siRNA (si-Axl1 or si-Axl2), cells were treated for 72 h with Crizotinib or Ceritinib at increasing concentrations. The evaluation of cell growth inhibitory effects was done by MTS as described in Section 2. The IC<sub>50</sub> values were calculated using Prism 5 (Version 5, GraphPad Software, Inc.).

efficiency was examined by Western blot (data not shown). As shown in Table 1, Axl depletion increased the sensitivity of ALK-mutated NB1643 cells to crizotinib, with 4- and 7-fold decrease in the  $IC_{50}$  values relative to si-Control cells. Similar results were observed in Kelly and SHSY5Y cells. The Kelly si-Axl knockdown cell lines were significantly more sensitive to crizotinib ( $IC_{50}$  = 60.5 and 32.6 nM) compared with Kelly si-Control cells ( $IC_{50}$  = 258.3 nM); and there was 7- and 13-fold increase in the sensitivity of the SHSY5Y si-Axl cells ( $IC_{50}$  = 72.0 and 41.9 nM) in comparison with the SHSY5Y si-Control cells (538.3 nM). In response to treatment with ceritinib, obvious decrease in the  $IC_{50}$  were seen in the si-Axl1 and si-Axl2-treated NB1643 (4- and 6-fold), Kelly (5- and 7-fold) and SHSY5Y (13- and 19-fold) cells, relative to respective si-Control cells. Additionally, depletion of Axl also sensitized cells to crizotinib or ceritinib treatment in NSCLC H2228 cells, which harbored the EML4-ALK fusion gene and also expressed Axl protein. However, little or no such changes were observed in Axl-negative NBSD cells. Altogether, these results suggesting the effectiveness of Axl inhibition in targeted therapy against ALK-driving cancers with ALK inhibitors.

### 3.4. Axl inhibitor promotes the apoptosis induced by ALK-TKIs

To further explore the application of Axl-specific small-molecule inhibitor in ALK-targeted therapy, we treated cells with BGB324 (an Axl inhibitor) in the presence of crizotinib (Fig. 3A) or ceritinib (Fig. 3B), and the frequency of apoptosis was measured by Annexin V binding assay. As each drug alone showed a modest effect, the combinatorial regimen exhibited higher frequency of apoptosis in Axl-expressing NB1643 (R1275Q) and SHSY5Y (F1174L) cells (Fig. 4A and B). Additionally, it was demonstrated that BGB324 had no effects on Axl-negative NBSD (F1174L) cells, also failed to increase the antitumor effects of crizotinib and

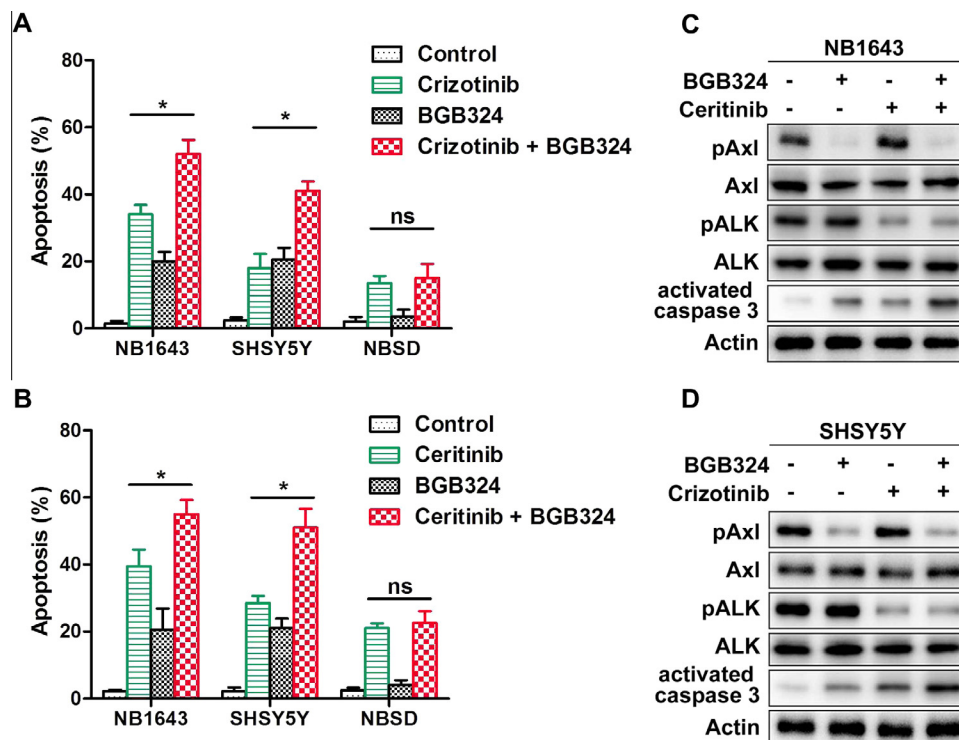
ceritinib. These results were further confirmed on a molecular level, the activities of Axl and ALK as well as the status of the apoptosis signaling effectors caspase3 were analyzed by Western blot. As shown in Fig. 3C, phosphorylated Axl and ALK were effectively suppressed by BGB324 and crizotinib respectively in NB1643 cells. Notably, combination treatment resulted in marked inhibition of the activities of Axl and ALK, as well as more efficiency in induction of activated-caspase3 than either single-drug alone, consistent with the increased apoptosis observed in the annexin V binding assay (Fig. 3A). Similar results were observed in the SHSY5Y cell lines (Fig. 3D), with combination treatment showing marked inhibition of phosphorylated Axl and ALK, as well as increased caspase3 cleavage.

### 3.5. Axl inhibitor enhances the efficacy of ALK-targeted therapy in vivo

A nude mice model was established to explore the efficacy of Axl inhibitor against NB *in vivo*. As shown in Fig. 4A, while treatment with crizotinib could partly postpone tumor growth, addition of BGB324 dramatically inhibited their growth. A significant delay in the tumor growth was also observed in the mice received treatment with BGB324 alone. Similar results were obtained by measuring the tumor weight (Fig. 4B). Additionally, while crizotinib and BGB324 could effectively suppressed the activities of ALK and Axl respectively, their activities were simultaneously blocked by combination treatment (Fig. 4C), consistent with the data *in vitro* experiment (Fig. 3C).

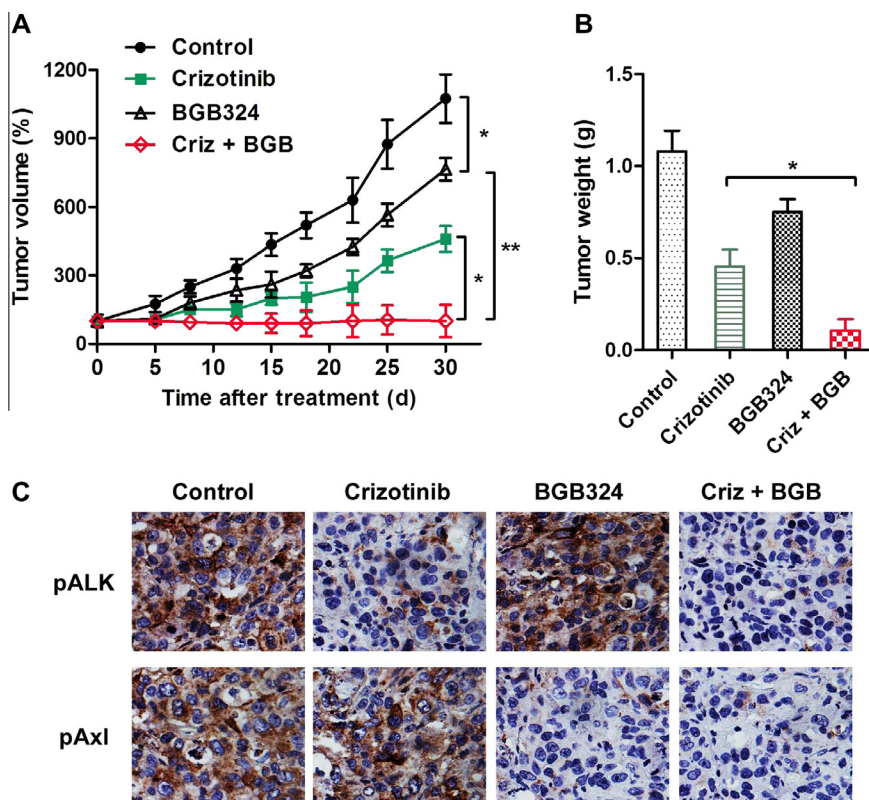
## 4. Discussion

Activating mutations in the oncogene ALK have been considered as the leading cause for most cases of hereditary neuroblastoma, providing the first tractable molecular target in this disease [2].



**Fig. 3.** Axl inhibitor promotes the apoptosis induced by ALK-TKIs. (A) After treated with crizotinib (100 nM), BGB324 (300 nM) or their combination for 48 h, cell apoptosis was determined by flow cytometry. (B) The induction of apoptosis by ceritinib (100 nM), BGB324 (300 nM) or their combination was examined. (C and D) Cells were treated with crizotinib (100 nM), BGB324 (300 nM) or their combination for 48 h, then whole-cell lysates were collected and the levels of indicated protein were analyzed by Western blot in (C) NB1643 and (D) SHSY5Y cells. \* $P < 0.05$  for the indicated comparisons; ns, not significant.





**Fig. 4.** Axl inhibitor enhances the efficacy of ALK-targeted therapy *in vivo*. (A) Nude mice carrying xenograft tumors were treated with crizotinib (50 mg/kg), BGB324 (125 mg/kg), or their combination. Tumor volume was measured. (B) At the end of treatment, tumors were removed and their weight was measured. (C) The expression of phosphorylated ALK and Axl in xenograft tumors was examined by IHC. \* $P < 0.05$  and \*\* $P < 0.01$  for the indicated comparisons.

The development of targeted therapy based on selective small-molecule inhibitors of ALK in neuroblastoma has advanced rapidly in preclinical and early phase clinical. Although tyrosine kinase inhibitors (TKIs) targeting ALK, including crizotinib and ceritinib, have showed excellent treatment effect in preclinical models, their clinical efficacy will ultimately be hindered by drug resistance [15]. It has been generally considered that the appearance of resistance to targeted therapy against diverse tumors is now considered largely inevitable that it offers much of a barrier for patients to benefit from such treatment [16].

In this study, we found that ALK was co-expressed with Axl tyrosine kinase in both NB tissues and cell lines. Various lines of evidence have demonstrated that Axl is expressed at abnormally high levels in a variety of malignancies, including various tumors [17,18]. Since activation of Axl by its ligands, such as GAS6, could result in strong upregulation of anti-apoptotic signaling pathways thereby promoting survival of tumor cells [11], further analysis was performed to determine the role of Axl activation in ALK-targeted therapy. As expected, Axl can be dramatically activated by GAS6 in the Axl-expressing NB cells (Fig. 2A). While crizotinib suppressed the growth of ALK-mutated NB cells, addition of GAS6 greatly rescued such suppression (Fig. 2B and C), indicating that ligand-activation of Axl may mediate insensitivity to ALK-TKIs.

Further study showed that inhibition of Axl by gene-silencing obviously increased the sensitivity to ALK-TKIs, crizotinib and ceritinib, in NB cells co-expressing Axl and ALK. Similar effect was also found in H2228 cells, a NSCLC cell lines carrying EML4-ALK rearrangement, implying the effectiveness of such strategy in other ALK-driving tumors. The cell apoptosis was further examined in NB cells after dual inhibition of ALK and Axl with inhibitors. BGB324 (R428), an inhibitor of Axl [19], could also elicit a certain

degree of apoptosis and a delay in tumor growth, suggesting the intrinsic effects of Axl in regulation of survival signaling in ALK-mutated NB. While ALK inhibitors partly triggered cell apoptosis, a higher degree of apoptosis was induced when in combined with inhibitor of Axl, consistent with the results as elucidated by Western blot that dual inhibition lead to inhibition of both ALK and Axl activation as well as induction of activated-caspase3. Furthermore, the initial experiment *in vivo* verified the antitumor effects of the combination treatment regimen based on inhibitors of ALK and Axl.

In summary, our work elucidated activation of Axl in ALK-mutated neuroblastoma may be involved in the drug resistance to ALK-targeted therapy based on TKIs. Inhibition of Axl promoted the antitumor effects of ALK inhibitors, at least partially through induction of apoptosis. Axl inhibitors may represent additional treatment options in specific patient populations.

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